



Peracetic acid is a suitable disinfectant for recirculating fish-microalgae integrated multi-trophic aquaculture systems

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ABSTRACT

Integrated multi-trophic aquaculture (IMTA) is a promising direction for the sustainable development of aquaculture. Microalgae have good potential to be integrated with recirculating aquaculture systems because they can use the nitrogen excreted from fish and share the same optimal pH value as in aquaculture. As a byproduct, the microalgae biomass can be used for fish feed or biofuel. However, the recirculating fish-microalgae IMTA system is under constant threat from fish pathogens and phytoplankton-lytic bacteria. Therefore, it is necessary to apply proper disinfectants as prophylaxis or treatment which are effective against these threats, but safe to fish and microalgae. For this purpose, peracetic acid (PAA) is a valid option because it is highly effective against fish pathogens and bacteria at low concentrations and degrades spontaneously to harmless residues. In the present study, we exposed the culture of a marine microalgae *Tetraselmis chuii* once per day for four days to four PAA products with differing hydrogen peroxide (H_2O_2)/PAA proportions at two concentrations (1 and 2 mg L^{-1} PAA). The H_2O_2 solutions at equivalent total peroxide ($H_2O_2 + \text{PAA}$) concentrations were tested in parallel. The results show that the growth and photosynthesis of *T. chuii* were not affected by three of the PAA products (Wofasteril® E400, Wofasteril® E250 and Applichem® 150) and equivalent H_2O_2 solutions at both concentrations. In contrast, Wofasteril® Lspez and an equivalent H_2O_2 solution at both concentrations caused irreversible culture collapse, photosynthesis dysfunction and irreversible cell damage. In conclusion, PAA products with low proportions of H_2O_2 are optimal disinfectants for fish-microalgae IMTA systems.

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1. Introduction

The growing world population has an increasing demand for high quality proteins. Based on the estimation of Tilman and Clark (2014), the global average dietary composition of fish and other seafood will increase 82% from 2009 to 2050. Increasing the world's supply of fish relies mainly on increasing aquaculture production, since the catch from the fisheries industry has been declining since the late 20th century (Pauly and Zeller, 2016). As discussed by

Naylor et al. (2000), many traditional aquaculture practices cause environmental and/or biological pollution and threaten fisheries by the use of fish oil and/or fish meal; they suggested that only sustainable aquaculture could help increase the supply of fish without harming natural ecosystems or fisheries. One option of sustainable aquaculture is the integrated multi-trophic aquaculture (IMTA) system.

Many IMTA systems have been developed and tested. For instance, an aquaponics system is the combination of aquaculture and hydroponics; it uses the waste from aquaculture as the fertilizer for hydroponics (Graber and Junge, 2009). However, hydroponics needs lower pH than aquaculture, so the water from aquaculture must be acidified and buffered. This results in higher technical and management costs and a limited recycling of nutrients. In contrast to plants, many algae species grow at the same pH

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value as fish. Therefore, algae have better potential to be integrated with aquaculture. Cahill et al. (2010) demonstrated that seaweed (macroalgae) performed better at removing ammonia and nitrite from a recirculating aquaculture systems (RAS) than traditional biofilm filtration (i.e., nitrifying bacteria). Gilles et al. (2014) developed an IMTA system consisting of microalgae, plankton and detritivorous/zooplanktivorous fish to replace the filtration unit of a RAS; they observed that an increase of the microalgae biomass led to a decrease of the total ammonia nitrogen. The studies detailed above demonstrate that algae are suitable to be used in the denitrification process of recirculating IMTA systems; as an added benefit, the algae biomass can also be harvested as a byproduct. The algae biomass, especially the microalgae biomass, can be used to produce biofuel (Lardon et al., 2009) or as a sustainable feed for shellfish, fish larvae and live prey (zooplankton) in aquaculture (Hemaiswarya et al., 2011).

The recirculating fish-microalgae IMTA system has several integrated problems. Fish may become infected by various pathogens. Meanwhile, the microalgae are threatened by phytoplankton-lytic bacteria, which can lead to reduced growth and even collapse of the population (Wang et al., 2013). To control fish pathogens, disinfectants can be regularly used as prophylaxis or treatment; however, the use of disinfectants might harm the microalgae culture. Therefore, it is important to find a disinfectant that can effectively control fish pathogens but not harm microalgae. To our knowledge, research has not been undertaken to find a suitable disinfectant for a recirculating fish-microalgae IMTA system.

Peracetic acid (PAA) has been recognized to be a sustainable disinfectant in aquaculture. It degrades completely within several hours after application (Pedersen et al., 2009) and results in harmless residues (Kitis, 2004). Noteworthy, the effective concentration of PAA is less than 2 mg L^{-1} against various pathogens (Pedersen et al., 2013). In contrast, another sustainable disinfectant, hydrogen peroxide (H_2O_2), needs a much higher concentration (over 20 mg L^{-1}) to achieve successful disinfection (Schmidt et al., 2006). Because toxicity highly depends on the dose, PAA has higher potential to fit in the recirculating fish-microalgae IMTA system. The aim of the present study is to test whether PAA at effective disinfection concentrations (up to 2 mg L^{-1}) is toxic to the microalgae cultivation in controlled lab conditions, and thus to determine whether PAA is a suitable disinfectant for recirculating fish-microalgae IMTA systems.

There are many commercial PAA products available and these products are equilibrium-mixtures of PAA, H_2O_2 and water with various H_2O_2 /PAA proportions. If these products are applied at the same PAA concentration, the difference in H_2O_2 concentration between the products would result in different acute toxicity values (Liu et al., 2015). Based on our past research, we hypothesize that there will be a difference in toxicity to microalgae with various commercial PAA products and we predict that products with a lower H_2O_2 /PAA proportion will be less toxic; consequently, they will be more suitable for prophylaxis/treatment in a fish-microalgae IMTA system.

2. Materials and methods

2.1. Chemicals

We tested four commercial PAA products with various PAA and H_2O_2 concentrations. Three of the products were Wofasteril® PAA formulations (Kesla Pharma Wolfen GmbH, Greppin, Germany): E400 (40% mass/volume [m/v] PAA, 12% m/v H_2O_2), E250 (25% m/v PAA, 30% m/v H_2O_2), and Lspez (3% m/v PAA, 40% m/v H_2O_2); the fourth product was Applichem® 150 (AppliChem GmbH, Darm-

stadt, Germany; 15% m/v PAA, 24% m/v H_2O_2). All other chemicals used were analytical grade and purchased locally.

2.2. Algae culture

A *Tetraselmis chuii* culture of the strain SAG 8-6 was obtained from the University of Göttingen (Göttingen, Germany) and cultured in 100-mL Erlenmeyer flasks with F/2 medium. The F/2 medium was prepared as described by the Canadian Phycological Culture Center at the University of Waterloo, Canada (<https://uwaterloo.ca/canadian-phycological-culture-centre/cultures/culture-media/f2>). The F/2 medium was sterilized by passing it through a Sartolab-P20® 0.2 μm micro-filter (Sartorius Lab Products & Services, Göttingen, Germany).

Flasks containing the algae culture were placed on a GFL 3005 shaker (GFL mbH, Burgwedel, Germany) in a KBW 720 incubator (Binder GmbH, Tuttlingen, Germany). The rotation rate of the shaker was $180 \times \text{g}$ and the temperature in the incubator was 18°C . There were two parallel OSRAM L 18W/865 daylight fluorescent lights (OSRAM GmbH, Munich, Germany) 0.5 m above the shaker, providing a light intensity of 2000 to 2700 lx on the surface of the shaking plate; the light cycle was controlled by a timer and set at 16 h light/8 h dark. Before toxicity experiments began, the stock algae culture was acclimated for 2 months under these conditions and the F/2 medium was refreshed once per week.

All work with the algae, including refreshing the medium, translocation and treatments were done under sterilized conditions. All materials that had contact with algae were sterilized by autoclaving or a hot air sterilizer.

2.3. Measuring PAA and H_2O_2 concentrations

The DPD (N, N-diethyl-p-phenyldiamine sulfate salt) photometric method was used to determine PAA and H_2O_2 concentrations based on a linear relationship between concentration and absorption (Liu et al., 2015).

The absorption-concentration relationship was determined by measuring the 550-nm absorption of solutions containing only H_2O_2 in distilled water at the concentrations of 0.112, 0.224, 0.336, 0.448, 0.56, 0.672, 0.784 and 0.896 mg L^{-1} on a DR3900 spectrophotometer (Hach Lange GmbH, Düsseldorf, Germany). The 550 nm absorption of 0.147 corresponded to 1.0 mg L^{-1} PAA or 0.448 mg L^{-1} H_2O_2 , and the 550 nm absorption of 0.294 corresponded to 2.0 mg L^{-1} PAA or 0.896 mg L^{-1} H_2O_2 .

The DPD photometric method could not measure the H_2O_2 concentration in the 1 and 2 mg L^{-1} Lspez solutions because the absorption values were too high for the spectrophotometer to read. Therefore, we used an iodometric titration method to determine the H_2O_2 concentrations (Greenspan and MacKellar, 1948). In short, a 5-mL Lspez sample was added to 30 mL of an ice-cold 10% H_2SO_4 solution in an Erlenmeyer flask. Next, 3 drops of 0.025 mol L^{-1} Ferriin solution (Carl Roth, Karlsruhe, Germany) were added to the mixture and titrated with 0.1 mol L^{-1} Ce^{+4} sulfate solution (Bernd Kraft GmbH, Duisburg, Germany) until the color turned from orange to blue. According to the method, 1 mL consumption of the Ce^{+4} sulfate solution corresponded to $1.701 \text{ mg H}_2\text{O}_2$.

2.4. Treatment and sampling

A volume of 200 mL of the stock algae culture was transferred to a 3-L Erlenmeyer flask and combined with F/2 medium for a total volume of 1.6–1.7 L. The flask was then placed in the incubator at 18°C for 72 h to allow the algae to reach exponential growth phase. Next, 30 mL of the algae culture was transferred into 51 100-mL Erlenmeyer flasks. The flasks were immediately divided randomly into 17 groups consisting of a control treatment and 16

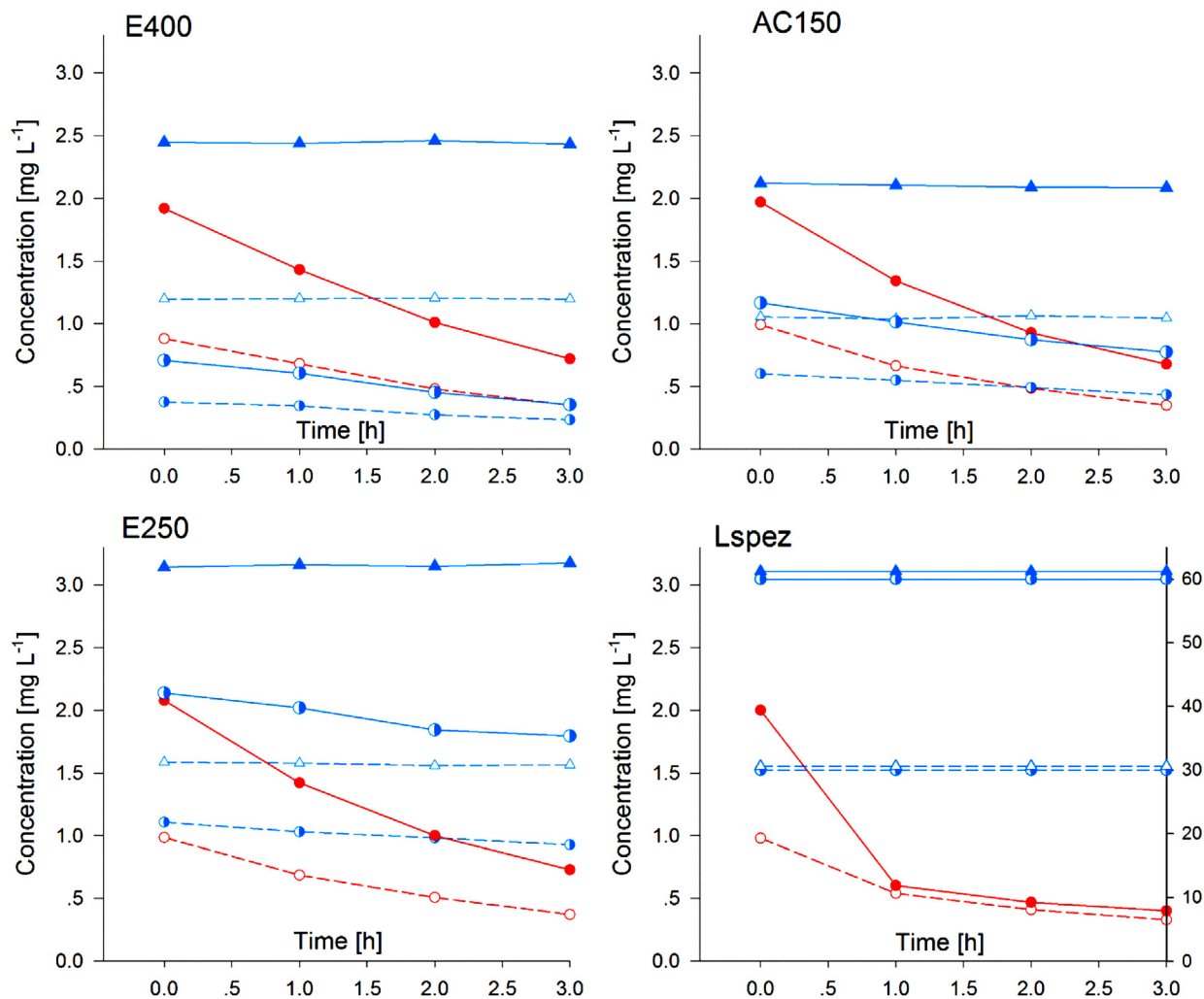


Fig. 1. Degradation of commercial PAA products and H_2O_2 solutions at equivalent total peroxide ($\text{H}_2\text{O}_2 + \text{PAA}$) concentrations over 3 h in F/2 medium at 18°C . Legend: Circles (\circ/\bullet) = PAA concentrations in treatments, half-filled circle (\bullet) = H_2O_2 concentrations in treatments with respective PAA products, triangles (Δ/\blacktriangle) = H_2O_2 concentrations in treatments with H_2O_2 solutions at equivalent peroxide concentrations, dotted lines and hollow symbols = 1 mg L^{-1} PAA and respective equivalent H_2O_2 solutions, solid lines and symbols = 2 mg L^{-1} PAA and respective equivalent H_2O_2 solutions.

PAA or equivalent H_2O_2 concentration treatments ($n=3$). The control treatment was exposed to aliquots of F/2 medium, while the treatments were then exposed to aliquots of the E400, E250, Lspez and AC150 products at PAA concentrations of 1 and 2 mg L^{-1} , as well as H_2O_2 solutions at equivalent total peroxide concentrations as each product. The experiment was initiated by mixing a 15 or $30 \mu\text{L}$ aliquot of F/2 medium, the diluted PAA products or equivalent H_2O_2 solutions into the respective flasks. We repeated the treatments every 24 h and retained a 2-mL sample before each exposure for growth and photosynthesis analyses. To equalize the light condition for each sample, the location of each Erlenmeyer flask on the shaker was randomly changed four times per day during the light period. Additionally, we measured the 3 h degradation of PAA and equivalent H_2O_2 concentrations for all treatments in algae-free F/2 medium.

2.5. Assessing the proportional change and cell density of the *T. chuii* culture

The *T. chuii* culture was assessed by measuring the absorption and directly counting the cell density with a Roche Innovatis/Schaefer System CASY TTC Cell Counter & Analyzer System (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany).

The absorption method was based on the linear relationship between cell density and absorption. First, a wavelength scan (400–700 nm) of the absorption of the stock *T. chuii* culture was performed. The peak absorption was at 677 nm. The 677-nm absorption of *T. chuii* cultures with different dilution ratios were measured and confirmed a linear relationship between the 677 nm absorption and dilution ratios. Therefore, measuring the 677 nm absorption of a sample over a set period of time estimated the proportional change of the *T. chuii* density.

As a supplementary method, the cell counter was used to read cell density within a certain size range. A $100\text{-}\mu\text{L}$ algae sample was diluted in $9900 \mu\text{L}$ micro-filtered ($0.45 \mu\text{m}$) CASY[®] ton electrolyte solution (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, Germany) and immediately measured for cell density at a detection range of 0–20 μm . The particles in the size of 7–15 μm were counted as *T. chuii* cells. Each sample was automatically measured three times and the final cell density was the mean of the three measurements.

2.6. Measuring the photosynthesis of the *T. chuii* culture

The maximum quantum yield was chosen as the parameter to evaluate the photosynthesis of *T. chuii* (Zbigniew and Falkowski,

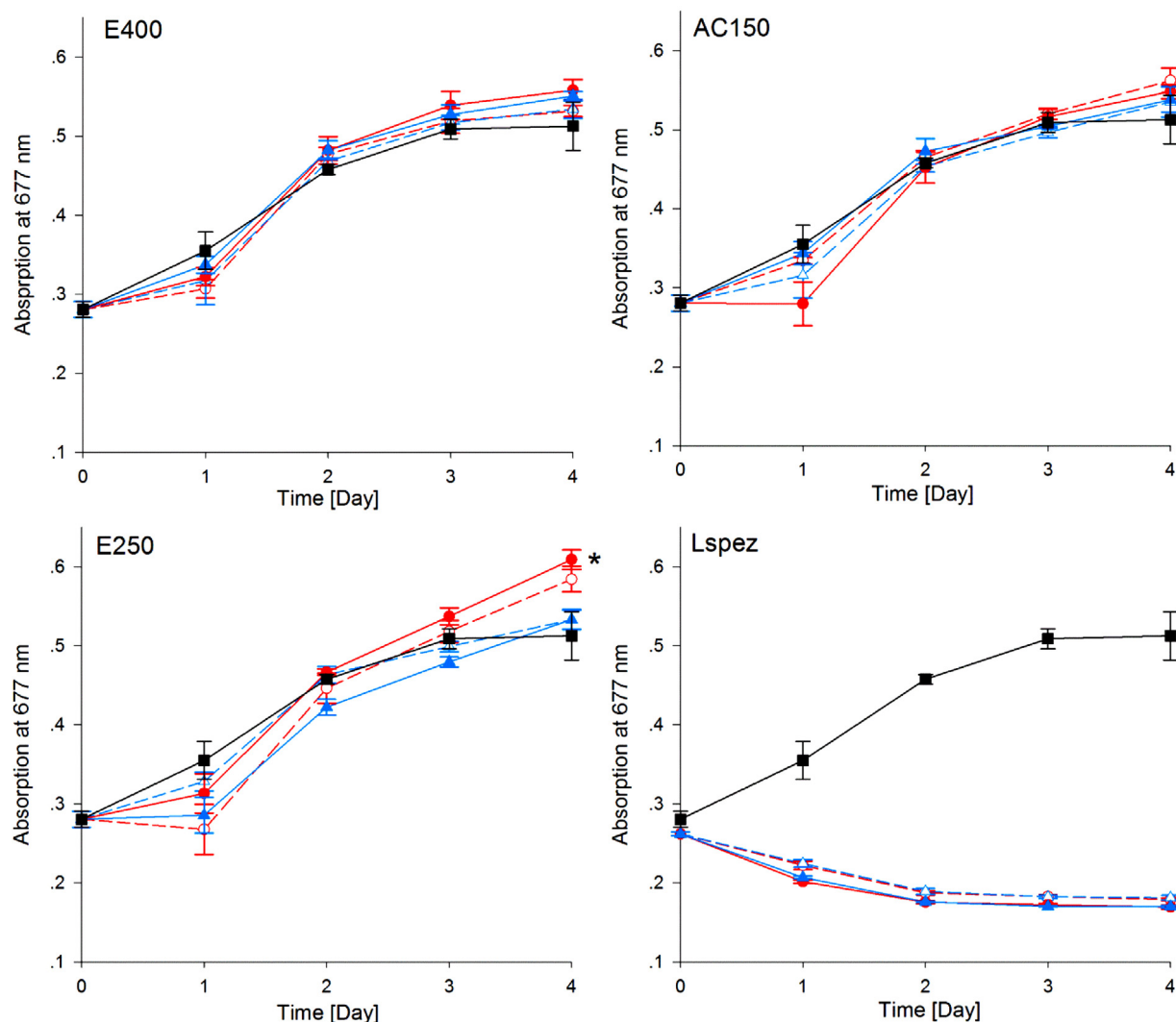


Fig. 2. Effect of the 1 and 2 mg L⁻¹ peracetic acid (PAA) products and H₂O₂ solutions at equivalent total peroxide (H₂O₂ + PAA) concentrations on the estimated proportional change of the density of the *Tetraselmis chuii* culture over 4 days at 18 °C. Light intensity = 2000–2700 lx. The PAA treatments were repeated every 24 h. Error bars indicate the standard error (n = 3). *, P < 0.05.

Legend: Solid square (■) and line = control, circles (○/●) = treatments with respective PAA products, triangles (△/▲) = treatments with H₂O₂ solutions at equivalent peroxide concentrations, dotted lines and hollow symbols = 1 mg L⁻¹ PAA and respective equivalent H₂O₂ solutions, solid lines and symbols = 2 mg L⁻¹ PAA and respective equivalent H₂O₂ solutions.

1993; Roháček, 2002). An Imaging-PAM M-series chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) in a dark room was used to measure the maximum quantum yield of the algae suspension. For the measurement, 1.6 mL of each algae sample was transferred to sterile 24-well plates. The plates were placed in the dark for 10 min and then slightly shaken before being aligned on the measuring platform of the fluorometer with the area of interest set to the center of each well. The maximum quantum yield (F_v/F_m) was automatically calculated and displayed after determination of minimal fluorescence yield (F_0) and maximum fluorescence yield (F_m). Three replicates were performed for each measurement and the mean value calculated for statistical analyses.

2.7. Statistics

The estimated proportional change of density, cell density and maximum quantum yield of *T. chuii* in the controls and treatments with PAA products and equivalent H₂O₂ concentrations were compared via one-way ANOVA with a Dunnett's test (two-tailed, $\alpha = 0.05$) on each day. In case of heterogeneity of variance, a ranked

ANOVA with Dunnett T3 test was performed instead. All statistical analyses were performed with SPSS® Statistics 21.0.0.1 (IBM Corporation, Chicago, USA).

The relationship between time and the estimated proportional change of density or cell density were fitted to a sigmoidal regression model, respectively. The curve fitting was performed with GraphPad Prism® 7 (GraphPad Software, Inc., California, USA). Akaike's Information Criterion (AIC) method was used to detect whether the curves of the treated and untreated samples fit to the same regression model.

3. Results

The H₂O₂/PAA proportion of the PAA products increased in the following order: E400 → AC150 → E250 → Lspez. In the Lspez product, the equivalent H₂O₂ concentration was around 30 mg L⁻¹ when the PAA concentration was 1.0 mg L⁻¹. The PAA degradation in the four commercial products at both dosages was generally over 50% within 3 h in algae-free F/2 medium, while H₂O₂ in PAA products

degraded less than 25%. There was no visible degradation in the flasks with 1.2 to 60 mg L⁻¹ equivalent H₂O₂ concentrations (Fig. 1).

The estimated daily proportional change of the density and cell density of *T. chuii* treated with three of the commercial PAA products (1 and 2 mg L⁻¹ treatments of E400, E250, AC150) was similar

to the control treatments and to the equivalent H₂O₂ concentration treatments (Figs. 2 and 3, $P < 0.05$). Meanwhile, their maximum quantum yields were similar (Mean values: 0.65–0.75, $P < 0.05$). One exception was the 2 mg L⁻¹ treatment of the E250 product that showed a higher estimated proportional change of density on

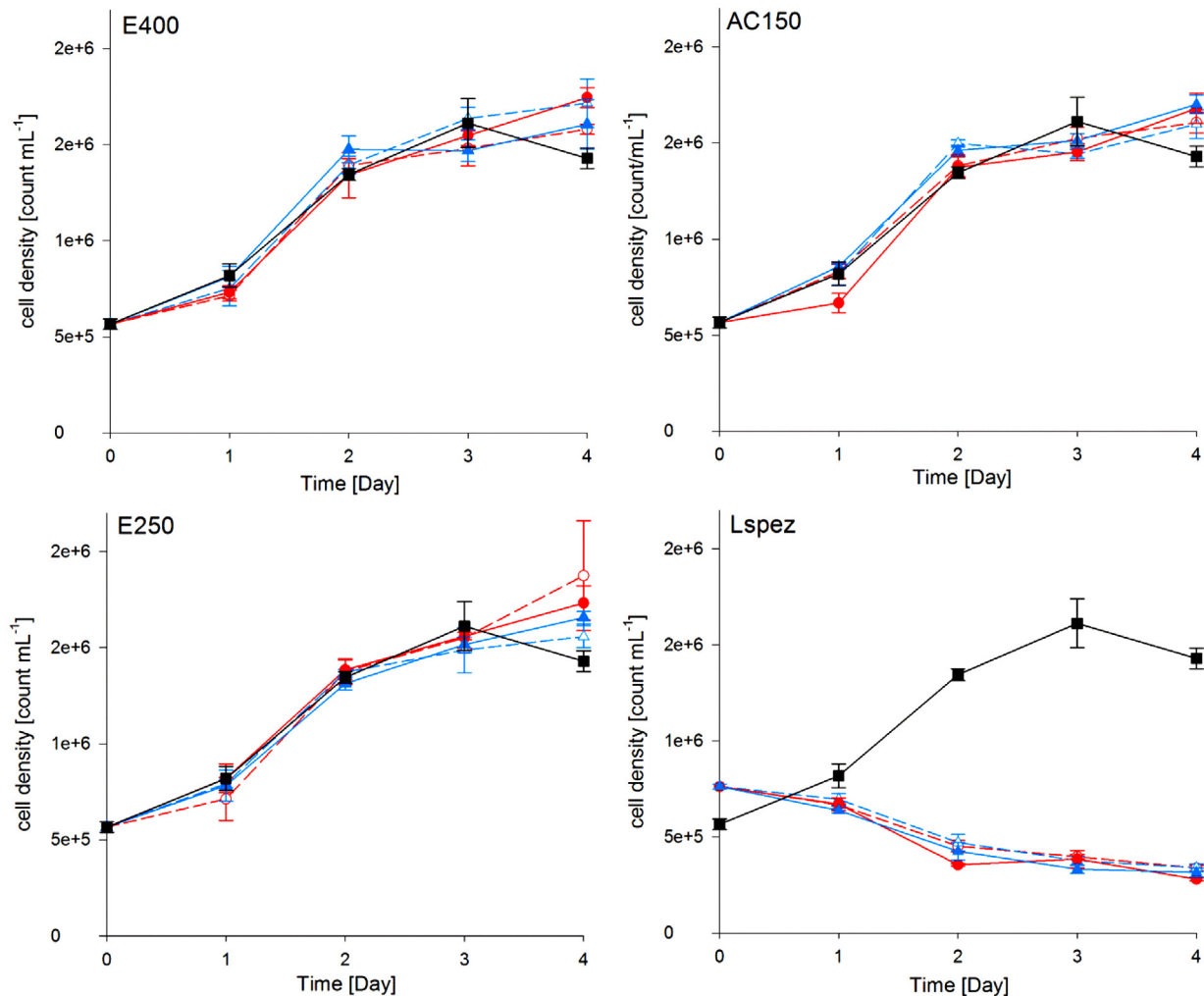


Fig. 3. Effect of the 1 and 2 mg L⁻¹ peracetic acid (PAA) products and H₂O₂ solutions at equivalent total peroxide (H₂O₂ + PAA) concentrations on the cell density of the *Tetraselmis chuii* culture over 4 days at 18 °C. Light intensity = 2000–2700 lx. The PAA treatments were repeated every 24 h. Error bars indicate the standard error ($n = 3$). Legend: Solid square (■) and line = control, circles (○/●) = treatments with respective PAA products, triangles (△/▲) = treatments with H₂O₂ solutions at equivalent peroxide concentrations, dotted lines and hollow symbols = 1 mg L⁻¹ PAA and respective equivalent H₂O₂ solutions, solid lines and symbols = 2 mg L⁻¹ PAA and respective equivalent H₂O₂ solutions.

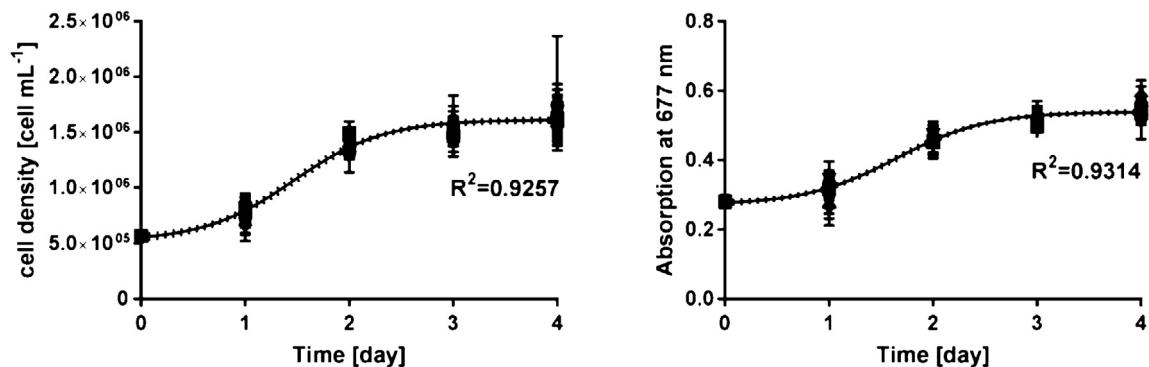


Fig. 4. The shared sigmoidal regression curve of cell density and estimated proportional change of the density (absorption at 677 nm) of PAA/H₂O₂-treated and untreated *Tetraselmis chuii* culture over 4 days at 18 °C. Light intensity = 2000–2700 lx. The PAA treatments were repeated every 24 h. Error bars indicate the standard error ($n = 3$). Confidence bands indicate 95% confidence interval of the true curve.

the 4th day as compared to the control treatment (Dunnett's test, $P=0.013$). Despite of that, the AIC method suggested that treated and untreated *T. chuii* culture probably shared the same regression model for cell density and estimated proportional change of density (Fig. 4). The probability was >99.99% and 99.96% for cell density and estimated proportional change of density, respectively. In contrast, the 1 and 2 mg L⁻¹ Lspez treatments and equivalent H₂O₂ concentration treatments significantly reduced the estimated proportional change of density and the cell density of *T. chuii*; this led to a constant zero value of maximum quantum yield after the 1st application. The *T. chuii* cultures in Lspez and equivalent H₂O₂ concentration treatments lost their green pigment (i.e., chlorophyll in the chloroplasts), and the individual cells lost their normal cell components and viability. The cultures were returned to the incubator without further disturbance, but showed no signs of recovery.

4. Discussion

The rapid degradation of PAA and slower degradation of H₂O₂ were measured in algae-free F/2 medium and indicated that the exposure duration of *T. chuii* culture to PAA was much shorter than to H₂O₂. Therefore, the toxicity of these commercial PAA products to *T. chuii* should initially consist of the concurrent effects of PAA and H₂O₂, and after the complete decay of PAA, the effect of H₂O₂ alone. Because the initial algae cultures were identical, the impact of algae on the degradation of PAA and H₂O₂ is comparable among treatments. In addition, the initial PAA concentrations of all PAA products were the same; consequently, the difference among treatments was due to their H₂O₂ concentrations. The equivalent H₂O₂ concentrations treatments showed little degradation in algae-free F/2 medium, so H₂O₂ had a longer contact time and a lower concentration decrease than PAA products. Consequently, the equivalent H₂O₂ concentration treatments should show stronger oxidizing effects. Despite this, the commercial E400, E250, and AC150 products and equivalent H₂O₂ concentration treatments showed no different effects on the growth and photosynthesis of *T. chuii*. This indicated the tested concentration of E400, E250, AC150 and equivalent H₂O₂ solutions were less than or equal to the no observed effect concentration (NOEC). In contrast, the 1 and 2 mg L⁻¹ PAA Lspez product and equivalent H₂O₂ (30 and 60 mg L⁻¹) led to irreversible cell death, culture collapse and undetectable photosynthesis; so their tested concentrations were above the absolute lethal concentration (LC₁₀₀). To fully understand the toxicity of PAA products to *T. chuii*, the concentrations between NOEC and LC₁₀₀ should be tested. Notwithstanding, the results of the present study are adequate to support further research into the practical use of commercial E400, E250 and AC150 products as disinfectants in fish-microalgae IMTA systems.

The mechanism of the toxicity might be that PAA and H₂O₂ are exogenous sources of reactive oxygen species (ROS). They can pass the cell membrane and increase the endogenous ROS concentration. Excessive endogenous ROS can induce programmed cell death (apoptosis) in algal cells (Segovia and Berges, 2009; Voigt and Woestemeyer, 2015) and contribute to the release of volatile organic compounds (Zuo et al., 2015). This explains the result that Lspez- and equivalent H₂O₂-treated *T. chuii* culture showed unrecoverable cell death.

In the present study, *T. chuii* showed stronger resistance to peroxide (in forms of PAA and H₂O₂), compared to fish pathogens (Pedersen et al., 2013) and daphnia (Liu et al., 2015). Many algae in the genus *Tetraselmis* were found to be able to degrade H₂O₂ because they produce antioxidant enzymes, such as superoxide dismutase, catalase and ascorbate peroxidase (Wong et al., 2003; Sigaud-Kutner et al., 2005). In fact, the ascorbate peroxidase universally exists in eukaryotic algae (Shigeoka et al.,

2002). Dimethylsulphoniopropionate (DMSP) and dimethylsulphide (DMS), which are generally present in marine algae, can scavenge hydroxyl radicals (Sunda et al., 2002). Apparently, the general antioxidant system of algae enables them to actively degrade oxidizers and reduce exposures that may harm their cell components. We suggest that the present study of *T. chuii* can be representative for marine microalgae. The commercial PAA products with low H₂O₂/PAA proportion, such as E400, E250 and AC150, should be safe to apply in aquaculture systems integrated by fish and any marine microalgae. Moreover, algae can assimilate ammonium, nitrite and nitrate and convert them to proteins or nuclear acids (Inokuchi et al., 2002; Mallick, 2002). In recirculating IMTA systems, algae have the potential to fulfill the nitrification function of the biofilter. Further research of applying PAA in recirculating IMTA systems and observing the performance of fish, algae and the biofilter is highly recommended.

5. Conclusion

We conclude that the commercial PAA products with a low H₂O₂/PAA proportion, such as E400, E250 and AC150 used in the present study, should be safe to use in recirculating fish-microalgae IMTA systems at PAA concentrations up to 2.0 mg L⁻¹. The PAA products with higher proportions of H₂O₂, such as Lspez used here, are not suggested to be used in recirculating fish-microalgae IMTA systems. Future studies should focus on applying PAA in recirculating fish-microalgae IMTA systems and observing the performance of fish, algae and the biofilter.

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